# EFFECT OF THIOUREA ON MOULTING AND PUPATION OF THE SILKWORM, BOMBYX MORI L.

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Insect blood, integument and other tissues contain the enzyme called phenoloxidase, or tyrosinase, which catalyzes the oxidation of various phenolic compounds to the corresponding quinones (for references see Sussman, 1949). Although there is much uncertainty about the role of phenoloxidase in the respiratory metabolism of insects, the function of phenoloxidase in the formation of insect cuticle is much better understood (*cf.* Mason, 1955; Hackman, 1958). Quinones, which are formed by the enzyme from phenols present in the cuticle, combine with proteins, thus forming the hard dark-colored layer of the cuticle. This process, called sclerotization and pigmentation, has been often investigated (Wigglesworth, 1947, 1948; Kawase, 1956). The view that phenoloxidase participates in the formation of insect cuticle is supported by numerous observations (Ito, 1953, 1954; Karlson, 1958; Kawase, 1960; Wojtczak, 1956), which show that the activity of the enzyme considerably increases during pupation and moulting, *i.e.*, at periods of intense cuticle-formation.

It seems thus interesting to investigate whether *in vivo* inhibition of phenoloxidase activity may affect moulting and pupation. Such attempts were first made by Dewitz (1901, 1902), who found that anoxia suppressed the hardening and the pigmentation of pupal cuticle of *Pieris brassicae*, and inhibited the pupation of *Lucilia caesar*. However, this effect, being rather unspecific, cannot be interpreted in terms of inhibition of the phenoloxidase alone. A more specific study on the inhibition of phenoloxidase was carried out by Fukuda (1953). He found that feeding silkworm larvae, *Bombyx mori* L., with mulberry leaves sprayed with thiourea produced serious disturbances both in larval moulting and in pupation.

We obtained similar results with a series of phenoloxidase inhibitors, including thiourea, injected into larvae of the waxmoth, *Galleria mellonella* L., and the silkworm, *Bombyx mori* L. (preliminary note: Wojtczak, 1954). The aim of the present investigation was to obtain more detailed information on the effects produced by an *in vivo* inhibition of phenoloxidase in silkworm larvae.

## Material and Methods

Larvae of a yellow strain of the silkworm, *Bombyx mori* L., obtained from the Institute of Sericulture in Milanówek, were used.

From 5 to 10 $\mu$moles of thiourea per gram body weight were introduced into the larvae by a microsyringe in the form of 0.2 *M* water solution. The total volume

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of the injected fluid was between 25 to 50 $\mu$l. per gram body weight. Control larvae were injected with the same volumes of 0.1 *M* KCl. In order to reduce bleeding the larvae were chilled before the injection and the wounds were sealed with paraffin.

The activity of phenoloxidase was measured, with catechol as the substrate, in homogenates of whole insects by the procedure described earlier (Wojtczak, 1956).

For histological examination fragments of the integument were taken from the mid-dorsal part of the larvae and pupae, fixed in Bouin's fixative, sealed in paraffin, cut on a microtome and stained with pyronine and methyl green, or with silver nitrate.

## Results

Injection of as much as 10 $\mu$moles thiourea per gram body weight had no immediate effect on the behavior of the larvae; they continued to feed or they spun normal cocoons. If the injection was made in the middle of the fourth or fifth instar, the larvae underwent normal larval moulting or pupation, respectively, and

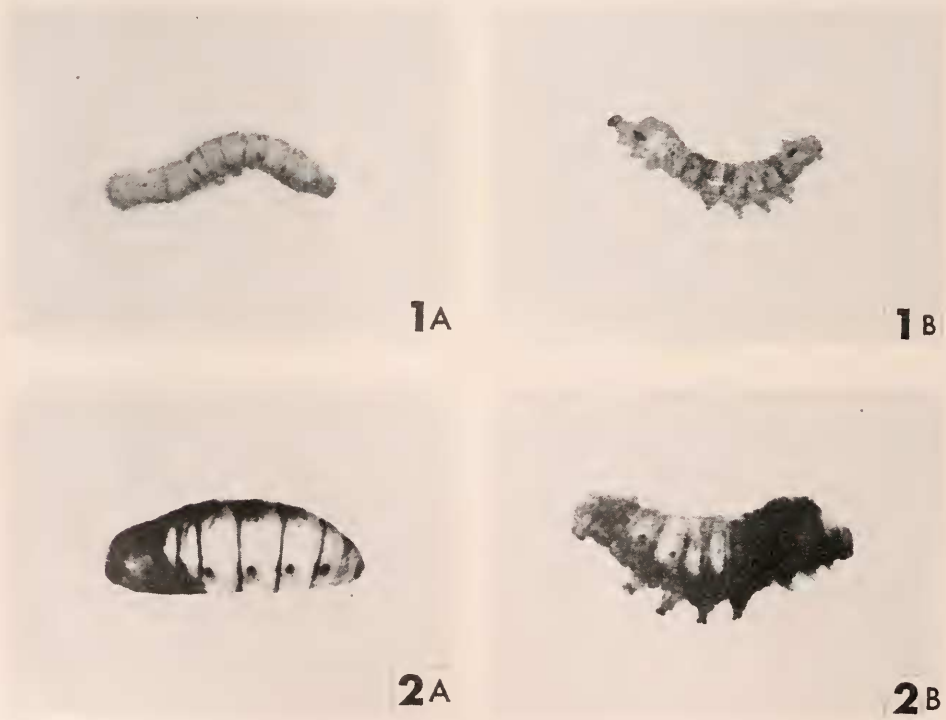



Figure 1. Effect of thiourea injected one day before the fourth moulting period. A, control larva (injected with KCl solution), 4 days after the injection, one day after moulting; B, larva injected with thiourea, same time after the injection; the old cuticle is retained and the swelling caused by an accumulation of fluid is visible at the dorsal side behind the head.

Figure 2. Effect of thiourea injected about one day before spinning. A, control (injected with KCl solution), 7 days after the injection, normal one-day-old pupa; B, insect injected with thiourea, same time after the injection; the old (larval) cuticle is retained.

TABLE I

*Effect of injecting thiourea into the fourth instar larvae on the activity of phenoloxidase and the behavior and moulting of the larvae. The larvae were injected with 10 μmoles thiourea per gram body weight; the control larvae received 5 μmoles KCl/g. body weight. The activity of phenoloxidase is expressed in ml. $O_2$ uptake per gram wet weight ($Q_{O_2}$) during the first 15 minutes of measurement*

| Time after injection | Control larvae | | Thiourea-injected larvae | |
|---|---|---|---|---|
| | Phenoloxidase activity | Behavior or state | Phenoloxidase activity | Behavior or state |
| 2 hours | 3.3 | Feeding | 1.0 | Feeding |
| 1 day | 3.0 | No feeding, immobile (beginning of the moulting period) | 1.3 | No feeding, immobile (beginning of the moulting period) |
| 2 days | 3.5 | Immobile | 0.9 | Immobile |
| 3 days | 4.1 | After ecdysis; start feeding | 0.7 | Swollen, the old cuticle retained, accumulation of haemolymph between the cuticles |
| 4 days | 3.6 | Feeding | 0.3 | Swollen, the old cuticle retained, accumulation of haemolymph |
| 5 days | | Feeding | | Dead |

they finally developed into normal adults. However, if the injection of thiourea preceded one day or less the beginning of the last larval moulting period,[2] or the beginning of the spinning, there was an inhibition of the moulting or pupation. In the case of the fourth instar larvae, the injected insects continued to feed and then stopped feeding exactly at the same time as control larvae. The initial phase of the moulting period, *i.e.*, the "moulting dormancy," also occurred normally. However, whereas one or two days later the controls underwent the normal ecdysis, the larvae injected with thiourea developed an abnormal appearance. The old cuticle was not removed. An abundant quantity of a haemolymph-like yellow fluid accumulated between the old and the new cuticles, causing a swollen appearance of the larva, especially at its anterior part (Fig. 1). The new cuticle appeared to be abnormally soft and delicate. In a few days the larvae died (Table I), and they did not survive even if the old cuticle was cut and removed experimentally.

When the fifth instar larvae were injected with thiourea approximately one day before spinning, they formed normal cocoons and turned into normal prepupae. However, they never pupated normally. The old cuticle was not removed and a great quantity of a haemolymph-like fluid accumulated between the old (larval) and the new (pupal) cuticle (Fig. 2). Whereas the cuticle of the control pupae turned brown within a few hours following pupation, the new cuticle of the injected insects remained pale and turned yellow or light brown only on the dorsal parts of the animal. Such abnormal insects survived sometimes up to 13 days, but never developed into adults.

[2] The term "moulting period" will be used in this paper to designate the ecdysis, or the removal of old cuticle, together with the preceding phase of immobilization ("moulting dormancy"). The last larval moulting period usually lasted two days.

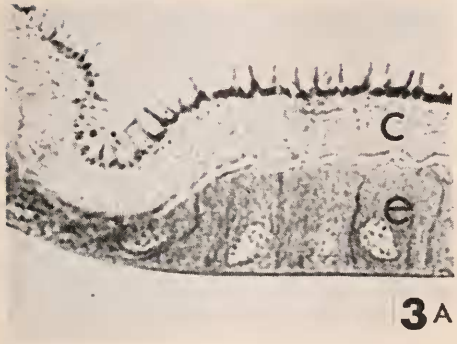
Table I shows the effect of the thiourea injection on the activity of phenoloxidase. The insects were homogenized at various times following the injection, and the activity of the enzyme was measured. In comparison with the control insects, the activity of phenoloxidase in the thiourea-injected animals was found to be strongly inhibited.

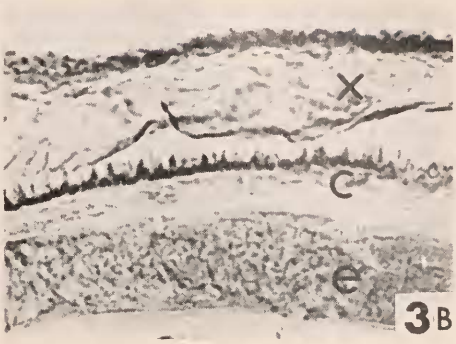
The character of these disturbances in the larval moulting and pupation caused by thiourea suggested that this compound might affect the formation of the new cuticle in such a way that it becomes more delicate and permeable to the haemolymph or, in the case of pupation, by preventing or inhibiting the sclerotization. To investigate this effect of thiourea in more detail, histological examination of the cuticle was carried out. It appeared (Fig. 3) that in the case of the fourth larval moulting no changes could be observed in the cuticle of thiourea-injected larvae as compared with the controls. The only difference was the presence of the old cuticle in the sections from thiourea-injected larvae (Fig. 3B). In the larvae injected with thiourea, as well as in the control larvae injected with KCl or not injected at all, the new cuticle developed simultaneously and appeared similar in thickness, shape and staining properties.

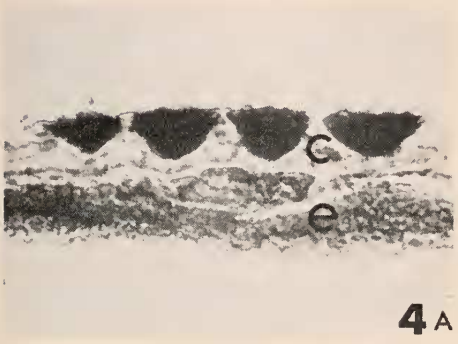
TABLE II

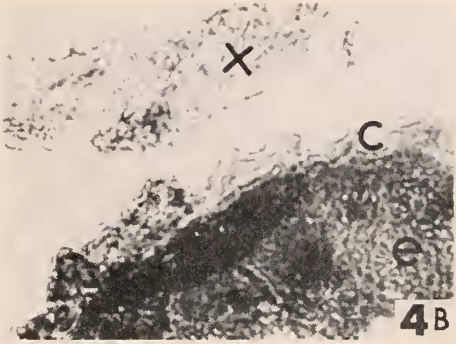
*Effect of injecting thiourea into the fifth instar larvae. All details as in Table I*

| Time after injection | Controls | | Injected with thiourea | |
|---|---|---|---|---|
| | Phenoloxidase activity | Behavior or state | Phenoloxidase activity | Behavior or state |
| 1 hour | 0.3 | Walking | 0.2 | Walking |
| 1 day | 0.8 | Spinning | 0.1 | Spinning |
| 2 days | 2.0 | Cocoon partly formed | 0.3 | Cocoon partly formed |
| 3 days | 3.3 | Cocoon completed | 0.3 | Cocoon completed |
| 4 days | 2.0 | Prepupa | 1.2 | Prepupa |
| 5 days | 3.0 | Prepupa | 0.5 | Swollen prepupa |
| 6 days | | Young (yellow or light brown) pupa | | Swollen prepupa |
| 7 days | | Pupa (dark brown) | | Swollen prepupa, accumulation of haemolymph between the cuticles |
| 8 days | | Pupa (dark brown) | | Swollen prepupa, accumulation of haemolymph between the cuticles |
| 9 days | | Pupa (dark brown) | | Swollen prepupa, accumulation of haemolymph between the cuticles |
| 10 days | | Pupa (dark brown) | | Swollen prepupa, accumulation of haemolymph between the cuticles |
| 11 days | | Pupa (dark brown) | | Swollen prepupa, accumulation of haemolymph between the cuticles |
| 12 days | | Pupa (dark brown) | | Swollen prepupa, accumulation of haemolymph between the cuticles |
| 13 days | | Pupa (dark brown) | | Dead |





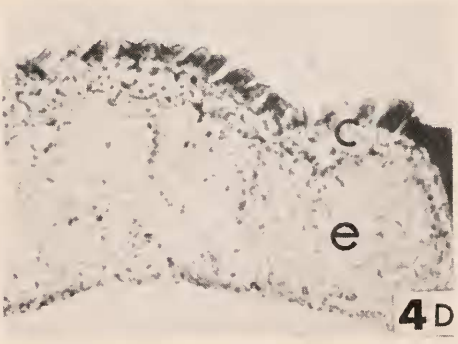



FIGURE 3. Effect of thiourea injection on the integument during the fourth larval moulting. A, control (larva injected with KCl solution), three days after the injection, shortly after moulting; B, larva injected with thiourea, same time after the injection. Indications: e, epidermal cells; c, cuticle; x, old cuticle (retained). Stained with pyronine and methyl green.

FIGURE 4. Effect of thiourea injection on the integument during pupation. A, control (injected with KCl solution), 6 days after the injection, shortly after pupation; black-coloured discs of sclerotized cuticle are clearly visible; B, insect injected with thiourea, 6 days after the injection; no sclerotization can be seen, the old cuticle is retained; C, control, 8 days after the injection of KCl solution, two days after pupation; note the thick and continuous layer of the sclerotized cuticle (black-colored); D, insect injected with thiourea, 12 days after the injection; sclerotization of the outer layer is not complete. Indications: e, epidermal cells; c, cuticle; x, old larval cuticle (retained). Stained with silver nitrate.

A quite different picture was seen in the case of the pupal moulting. Here, after injection of thiourea the inhibition of the sclerotization and pigmentation was clearly visible. In the untreated insects and in those injected with the KCl solution, the sclerotization was manifested by the formation of hard brown-colored discs in the epidermis (Fig. 4A), whereas no such discs could be seen at the same time in the insects injected with thiourea (Fig. 4B). The discs increased in size and finally joined together, forming a layer of brown cuticle staining black with $AgNO_3$ (Fig. 4C). In the insects treated with thiourea the formation of these brown discs was delayed for several days, and they were usually smaller and less numerous than in normal and control specimens. Very often, the discs did not appear at all; instead, the outer border of the epidermis turned brown, forming a thin layer of sclerotized cuticle (Fig. 4D).

## Discussion

The present investigation demonstrates that thiourea, when injected into silkworm larvae, disturbs the moulting process. This is manifested most conspicuously by the retention of the old cuticle. In this respect these results agree with earlier observations of Fukuda (1953) on silkworm larvae fed with mulberry leaves coated with thiourea.

Another effect of thiourea poisoning observed in the present investigation was the accumulation of haemolymph or a haemolymph-like fluid between the old and the new cuticles. Whether this was the result of tearing the new cuticle, rather than the result of filtration of haemolymph through the cuticle, cannot be decided as yet. A similar accumulation of a haemolymph-like fluid between the two cuticles was observed by Jeuniaux (1958) in larvae ligated behind the head. As is well known, ligation prevents hormones produced in the anterior part of the body (brain hormone and moulting hormone) from penetrating to the posterior part, thus preventing the moulting. It has been shown (Karlson and Schweiger, 1961) that the moulting hormone (ecdyson) increases the activity of phenoloxidase. Thus, it seems possible that the accumulation of a fluid between the two cuticles in the ligated larvae is due to some enzymatic disturbances, as in the case of the thiourea-injected larvae, rather than to the simple mechanical effect of the ligation, as suggested by Jeuniaux (1958).

As shown by the present investigation, thiourea had no visible effect on the processes of feeding and spinning, *i.e.*, during periods of a low phenoloxidase activity (Wojtczak, 1956). Abnormalities did not appear until moulting or pupation, *i.e.*, at those stages where there is a considerable increase in the activity of phenoloxidase (Wojtczak, 1956). This increase was found to be partly prevented by thiourea injected into the larval body. Thus, it seems highly probable that the abnormalities in larval moulting and pupation brought about by the injection of thiourea are mainly, if not solely, due to the inhibition of phenoloxidase.

The normal behavior of spinning larvae injected with thiourea observed in our experiments can be contrasted with the results of Jones and Wilson (1959), who found abnormalities in cocoon spinning by larvae of *Philosamia cynthia* injected with phenylthiourea. It cannot be decided whether these differences are due to a slightly different action of thiourea and its phenyl derivatives, or to a different susceptibility of *Bombyx mori* and of *Philosamia cynthia* to the poison used. It

would be also interesting to investigate whether, besides abnormalities in cocoon spinning of *Philosamia,* there were disturbances in the pupation process similar to those observed in the present study.

The fact that the new cuticle of thiourea-injected silkworms was more delicate and permeable to haemolymph, and that the process of sclerotization was inhibited indicate that thiourea may cause serious morphological changes in the cuticle. Such changes, consisting of a delay and a partial inhibition of the sclerotization and pigmentation, were indeed revealed by the present investigation in the case of pupal moulting. It cannot be excluded, however, that thiourea may also induce changes and abnormalities in other organs and tissues of the insect.

In insects injected with thiourea, not only was the sclerotization of the cuticle partly inhibited, but also the cuticle itself was usually thinner than in the control insects. On the other hand, the layer of epidermal cells appeared much thicker.

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## Summary

1. Thiourea injected into silkworm larvae, *Bombyx mori* L., was found to inhibit the activity of phenoloxidase.

2. The effect of thiourea injection on moulting and pupation of the larvae was examined. When the injection preceded 24 hours or less the fourth moulting period or the spinning, disturbances in larval moulting and in pupation were observed, respectively. They consisted of a retention of the old cuticle and an accumulation of a haemolymph-like fluid between the old and the new cuticles. Histological examination revealed a partial inhibition and a delay of the sclerotization of pupal cuticle.

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